Effect of ErbB4 on Triple Negative Breast Cancer Cell Growth and Migration

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Dedication

I dedicate this work to all research-scientists around the world working tirelessly to help advance the field of medicine and improve the quality of our patients' lives.

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Abstract

Members of the ErbB subfamily of receptor tyrosine kinases are critical regulators of normal mammary gland development, and alterations in their signaling have been associated with breast tumorigenesis. ErbB4 expression in breast carcinomas predicts improved patient survival and inversely correlates with tumor grade, metastasis and disease recurrence. When examined in the context of the breast cancer molecular subtypes, ErbB4 expression is rarely expressed in the triple-negative tumor subtype, which is associated with poor prognosis. Recently, our lab discovered a genomic context for the loss of ErbB4 expression in metastatic, refractory triple-negative breast cancer (TNBC) samples by next generation sequencing technology. The goal of this study was to examine the effects of ErbB4 overexpression on the growth and migration of TNBC cell lines. A GFP-containing construct was used to overexpress ErbB4 in the ErbB4-negative TNBC cell lines BT-20, BT-549 and MDA-MB-468. An empty vector construct was used as the control. Expression was confirmed by western blot and fluorescence microscopy to detect expression of ErbB4 or GFP respectively. Cell motility and growth was assessed with a transwell migration assay and a sulforhodamine B assay to measure cell density, respectively. Our data indicates that overexpression of ErbB4 resulted in no significant difference in the migration of BT-549 or MDA-MB-468 cells but resulted in a slight increase in the migration of BT-20 cells. ErbB4 had a growth inhibitory effect on BT-549 and BT-20 cells but showed no difference in the growth of MDA-MB-468 cells. This data suggests that multiple ErbB4-mediated mechanisms occur to alter the growth of TNBC cells. Although the translational significance of ErbB4 loss may be in its ability to predict outcome in patients with TNBC, more work is needed to elucidate the molecular mechanisms mediating its function.

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Introduction

Triple-Negative Breast Cancer

Breast cancer is the most common non-skin malignancy among women in the United States. Over 200,000 women were estimated to have been newly diagnosed with invasive breast cancer and approximately 40,000 patients died from their disease in 2010[1]. Triple-negative breast cancer (TNBC) describes an aggressive, heterogeneous subtype of breast cancer in which the tumor cells lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2). TNBC comprises 10-20% of all breast cancers with a disproportionately higher prevalence in younger, African American, and Hispanic women[2, 3, 6]. As a result of its distinct cellular phenotype, receptor-targeted therapy is not possible leaving chemotherapy as the standard treatment. Its aggressive clinical course, dismal prognosis and markedly limited treatment options have intensified current interest in this population.

The most widely used system to categorize breast cancer incorporates histomorphological data as well as TNM (i.e. tumor size [T], lymph node spread [N], and distant metastasis [M]) staging information[4]. Recently, gene expression studies, determined by DNA microarray experiments, have allowed breast cancer subgroups to be classified based on transcriptomic similarity[4, 5]. These highly reproducible subtypes include: luminal A and B, HER2+/ER-negative, normal breast-like, basal-like and potentially a claudin-low subtype[5]. Of these, the basal-like molecular phenotype is of particular interest as 70-80% of basal-like breast cancers (BBC) exhibit triple-negative receptor status and vice versa[4-10]. Although no uniformly agreed-upon definition for BBC currently exists, it is generally characterized by an absence or low levels of ER expression, an absence of HER2 overexpression, and a gene-expression profile that is similar to that of the basal–myoepithelial layer of the normal breast[6]. It is no surprise then that this class of tumors is characterized by aggressive growth and the worst patient outcomes[4]. Though the vast majority of TNBC cells display a basal epithelial phenotype, other documented molecular manifestations include the claudin-low variant, which describes tumor cells exhibiting stem cell-like features and epithelial-to-mesenchymal transition abilities, and the interferon-rich subgroup[6]. Notably,

tumors of the latter type are associated with relatively improved survival[6]. Studies have repeatedly demonstrated that the poorest prognosis appears to be primarily confined to TNBC tumors that express basal markers, i.e. cytokeratins or epidermal growth factor receptor (EGFR)[3]. A recent analysis of 4000 cases compared the prognostic value of the triple-negative receptor status with and without the addition of basal markers[7]. The poor prognosis of the triple-negative phenotype was determined almost entirely by those tumors positive for basal markers, and the inclusion of basal markers had in fact superior prognostic significance compared to depending on receptor expression alone[7]. Studies as these have helped to reveal the diversity that exists within TNBC. It appears likely that TNBC does not represent a single entity, but rather an assortment of different diseases sharing a common immunohistochemical status[6, 9].

Typical clinical features of TNBC include increased mitotic activity and atypical mitotic figures, high Ki67 index, marked cellular pleomorphism, high nuclear atypia, high nuclear-cytoplasmic ratio, scant stromal content, central necrosis or central acellular zones, multiple apoptotic cells, pushing margins of invasion and stromal lymphocytic infiltration[3, 5]. The vast majority, 50-80%, of these tumors present as infiltrating ductal carcinomas, not otherwise specified[4, 9]. Both BBC and TNBC have been associated with large tumor size, high tumor grade, lower 5-year survival rates, and increased likelihood of distant metastases[14]. In further highlighting the distinction of TNBC from other types of breast cancer, a case–control study carried out as part of a national mammographic screening program revealed that TNBC was over-represented among women with interval breast cancers[8], again indicating the rapid tumor growth seen in this subgroup. Additionally, TNBCs are less likely to metastasize to bone and more likely to metastasize to the viscera, particularly to the lungs and brain, compared to other forms of breast cancer[10].

Numerous studies have suggested unique demographic associations and risk factors for acquiring TNBC. For example, a cohort study of 1601 breast cancer patients, including 180 triple-negative cases, revealed a younger mean age at diagnosis for women with the triple-negative subtype (53 vs. 58 years, $P < 0.0001$)[11]. Further, in evaluating for differences of race, a separate, single institutional study of 2230 women demonstrated that the triple-negative

receptor status was more common in African–Americans compared with non-African–American women (20.8% vs. 10.4%, *P*< 0.0001)[12]. Similarly, in the population-based Carolina Breast Cancer Study (CBCS), 16% of non-African–American women were diagnosed with BBC (defined as TNBC plus cytokeratin 5 or EGFR positivity) compared with 26% of African–American women, and 24% of premenopausal women compared with 15% of postmenopausal women[13]. The subset of breast cancer patients most likely to have BBC are premenopausal African–American women in whom this subtype comprises 27–47% of breast tumors[5, 13]. Other associated populations include Hispanic and obese patients, and in all at-risk groups combined TNBC accounts for approximately 25% of breast cancer prevalence[15]. Furthermore, in contrast to the established risk factors for luminal disease, such as nulliparity and later age at first birth, risk for BBC is increased with higher parity and younger age at first term birth[3, 5, 6]. In addition, a lower lifetime duration of breastfeeding, earlier onset menarche, and increased abdominal adiposity also increased risk for BBC[3, 5, 6]. At the same time, these factors did not affect risk of the more common ER-positive (luminal A) breast cancer, with the exception of an elevated waist-to-hip circumference which represented a lesser risk factor for luminal A cancers in postmenopausal women[3, 5, 6].

With regards to treatment, the lack of tumor hormone receptors severely limits curative intervention. Intriguingly, there does exist a minority of women with triple-negative disease who have a pathologic complete response (pCR) to chemotherapy and thus an excellent outcome[16]. Though a precise definition of pCR has not yet been agreed upon, it is often characterized as the absence of invasive disease in the breast and axilla (ypT0/is ypN0)[17] with mounting evidence suggesting that it may act as a valid predictor of therapeutic endpoints such as disease-free survival, event-free survival and overall survival, particularly in HER-2 positive and TNBC[17, 18]. In a neo-adjuvant study involving 255 patients with triple-negative disease receiving either anthracycline or anthracyline and taxane-based regimens, the pCR rate of triple-negative tumors was significantly higher than that identified for the other subtypes (22% vs. 11%, *P* = 0.034)[3, 16]. In two similar neo-adjuvant trials comparing pCR rates in TNBC to luminal cancers the results were more dramatic (25-45% vs. 6-7%)[5, 17, 19, 20]. Those patients with pCR had a good prognosis; however, the outcome for the majority of TNBC who still have

residual disease after treatment is relatively poor, especially in the first three years where relapse is highest[5, 6, 11, 16].

ErbB4

The epidermal growth factor receptor (EGFR/ErbB1/HER1) family has long been implicated in various human cancers. It is made up of four receptor tyrosine kinases: ErbB1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. As such, these receptors generally follow the typical pattern of homo- or heterodimerization and autophosphorylation upon ligand binding to begin a complex, multi-layered signaling cascade. Exceptions to this sequence include the lack of kinase activity in ErbB3 and the absence of any known ligand for ErbB2, which contributes by serving as the preferred heterodimerization partner for the other receptors[21, 22]. The phosphorylation residues on the cytoplasmic domain serve as docking sites for adapter proteins that possess Src homology domain 2 (SH2) and phosphotyrosine binding (PTB) motifs[23, 24]. Several of the phosphorylated tyrosine sites can then bind unique effectors, including Ras, mitogen-activated protein kinase (MAPK), Src, signal transducer and activator of transcription (STAT) 3/5, phospholipase Cγ (PLCγ), protein kinase C (PKC), and phosphatidylinositide 3-kinase (PI3K)[23-26]. These effectors are typically coupled to increased survival, proliferation, motility and invasiveness displayed by malignant tumor cells[22, 23].

A number of ErbB-specific ligands have been discovered, each of which containing an EGF-like domain that grants binding specificity allowing them to be divided into three groups[22]. The first group of agonists binds only to ErbB1 and comprises of EGF, amphiregulin (AR), and transforming growth factor-α (TGF-α); the second group binds both ErbB1 and ErbB4 and includes betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR); and the third group is composed of the neuregulins (NRG) and forms two subgroups based upon their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4)[22, 23]. These ligands are expressed as integral membrane proteins and are cleaved into their final, soluble form by metalloproteinases, typically members of the ADAM (a disintegrin and metalloproteinase) family of membraneous proteases[23]. One intriguing feature of ErbB receptors is their delicate sensitivity, even when the same ligand is used, resulting in a vast

array of cellular responses[21, 22, 24]. For example, stimulation with NRG produces wide-ranging effects including cell proliferation, growth inhibition, differentiation and apoptosis of segments of a cell population[21]. The plethora of divergent responses observed between cell lines and at various stages in the cell cycle within the same cell line is unquestionably influenced by differences in the recruitment of signal transduction pathways[21, 27].

ErbB4 is the last member in this family of receptors to be discovered. A unique feature of ErbB4 among ErbB receptors is that ErbB4 pre-mRNA is alternatively spliced at either the extracellular or intracellular regions, generating structurally discrete juxtamembrane (JM-a and JM-b) and cytoplasmic (CYT-1 and CYT-2) isoforms[28, 29]. The JM-a isoforms, unlike the JM-b isoforms, contain proteolytic sites that are cleaved by TNF-α converting enzyme (TACE) and γ-secretase sequentially, releasing a soluble intracellular domain (4ICD) that is capable of coregulating transcription in the nucleus[28]. The CYT-1 isoforms, but not CYT-2 isoforms, have a 16-amino acid stretch that includes interaction motifs for intracellular signaling molecules, such as PI3-K[28]. 4ICD is characterized by multiple, diverse biological activities and cellular responses including differentiation of mammary epithelial cells and lactation, activation of pro-apoptotic pathways, cell cycle arrest, modulation of transcription through formation of complexes with transcription factors and cell proliferation[29, 31]. In addition, it has been demonstrated that these diverse responses are associated with the localization of 4ICD in different cell compartments[29-32, 38, 39]. Nuclear 4ICD functions as a potent ERα co-activator, directly interacting with ligand-associated ERα and promoting the proliferation of ERα positive breast tumor cells[29]. On the other hand, cytosolic 4ICD accumulates within mitochondria, promoting apoptosis of tumor cells through the activity of the cell-killing BH3 domain[29]. Hence, exploitation of 4ICD cell localization may serve as a potential therapeutic target in breast cancer patients[29].

One of the primary functions of ErbB4 *in vivo* is in the terminal differentiation of mammary glands during late-pregnancy and lactation induction[27, 33, 34, 40]. In addition, it plays a crucial role in the development and differentiation of cardiovascular and neural tissue[24, 25]. Studies have shown ErbB4 expression is detectable in less than half of breast cancers[29, 34]. Despite the known oncogenic potential of the EGFR family, the existing evidence suggests that ErbB4 is

characterized by antiproliferative and pro-apoptotic activity. In breast cancer patients, ErbB4 expression is associated with low proliferative indices, cell growth inhibition, a relatively well differentiated histopathological grade, hormone receptor positivity, increased survival, reduced recurrence, and has been found to antagonize the effect of ErbB2 on clinical outcome[28, 33]. All of these findings indicate a more favorable prognosis, supporting the protein's tumor suppressive role.

Our lab has recently performed whole-genome and transcriptome sequencing in TNBC samples. We demonstrated loss of ErbB4 expression in a high number of samples and, importantly, we identified multiple genomic mechanisms underlying the loss of ErbB4 expression. These include a homozygous deletion, point mutation and 21Mb chromosomal deletion, which breaks ERBB4. Due to our recent findings describing the genomic mechanisms associated with ErbB4 loss in a high number of TNBC samples, we predict that the loss of ErbB4 is detrimental to TNBC cells. Therefore, we hypothesize that overexpression of ErbB4 will lead to anti-neoplastic effects on TNBC cells. The goal of our study was to measure the growth and migration of TNBC cell lines after reintroducing ErbB4 into ErbB4-negative TNBC cell lines. The first aim was to exogenously express ErbB4 in TNBC cell lines. The second aim was then to measure the growth and migration of ErbB4-overexpressing TNBC cell lines. Ultimately, we strive to better understand the association of ErbB4 expression with TNBC biology and clinical manifestations.

Research Materials and Methods

Plasmid transfection

pCMV6-AC-GFP plasmid, containing the ERBB4 gene, was obtained from Origene Technologies. The plasmid was transformed in Top10 competent bacterial cells. The next day, several bacteria colonies were selected for insert screening. Large scale plasmid purifications were carried out using the Qiagen maxiprep kit. Empty vector controls are used for control purposes.

The TNBC cell lines used were BT-20, BT-549, and MDA-MB-468, all of which lack expression of ErbB4. All cell lines were cultured in media containing 10% fetal bovine serum. The cells were seeded the day prior to transfection to produce a starting cell confluency of approximately 60 percent. On the day of transfection, cells were replaced with fresh media and the X-tremeGENE HP DNA Transfection Reagent protocol was followed. Following incubation of at least 18 h, these cells were either lysed to run a western blot or counted to proceed with the growth and migration assays.

Growth assay

The sulforhodamine B (SRB) assay was used to assess cell density. The growth plates were seeded at a concentration of 1000 cells/well (5 cells/µl) and were left to incubate at 37°C. Six replicates of both the ErbB4-transfected and the empty vector-transfected cells were prepared for each cell line. Growth progression was measured at days 1, 3, 5, and 7 post-seeding. The cells were fixed on those days and eventually stained and solubilized before measurements were taken. A solution of 50% trichloroacetic acid (TCA) was used to fix the wells. The plates were then allowed to incubate for 1 h at 4°C before they were rinsed thoroughly in water and allowed to dry. After ensuring that the plates have completely dried, the SRB cell staining assay was performed using 0.04% SRB as the reagent. The plates were left at room temperature for 20 min before being thoroughly rinsed in a 1% acetic acid wash and allowed to dry. Lastly, after again ensuring dry wells, the cells were solubilized in 10 mM Tris solution. The wells were briefly agitated to ensure complete homogeneity, and the samples were analyzed with a spectrophotometer at an absorbance of 565 nm. From this data a graph was generated,

plotting the growth of each cell line over the week. This process was repeated to produce three trials.

Migration assay

The CytoSelect™ 96-well cell migration assay kit was used to assess cell motility. The sterile migration plate consists of three parts: a plate cover, membrane chamber, and a feeder tray on the bottom. 50,000 cells were seeded per well (500 cells/µl), and a sterile 0.5% bovine serum albumin (BSA) solution was also added to the cell suspension. No chemoattractant was used, rather each cell line's respective media was added to the wells of the feeder tray. Four replicates of each condition (ErbB4 or empty vector) were prepared for each cell line. The membrane chamber was then placed atop the feeder tray and the desired concentration of BSA and cell solution was added before allowing the plate to incubate at 37°C overnight. The following day, the cell detachment solution was added to the cell harvesting tray. After discarding the residual fluid in the membrane chamber, this chamber was placed into the cell detachment solution. Following 30 min of incubation at 37°C, a lysis buffer/dye solution was added to the mixture and the plate was read with a fluorescence plate reader at 480 nm/520 nm. The data generated by this machine similarly allows a graph to be plotted measuring migration of the cell lines, each compared to an empty control. This process was repeated to produce three trials.

Results

Western blot

To verify successful transfection and expression of ErbB4 in the three TNBC cell lines (BT-20, BT-549 and MDA-MB-468), Western blotting of ErbB4-transfected cells compared to empty vector controls was performed. Figure 2 reveals a thick band corresponding to ErbB4 protein visualization in the transfected cells, confirming a successful transfection. Further, the ErbB4 band is absent in the empty vector signifying an accurate negative control. For the positive control, α-tubulin was used and indeed was found present in both empty vector construct and ErbB4-containg TNBC cells.

SRB growth assay

In examining the effect of ErbB4 on TNBC cell growth and proliferation, the SRB growth assay protocol was followed recording measurements at day 1, 3, 5 and 7 post-seeding. Figure 3 illustrates via bar chart ErbB4-transfected versus empty vector TNBC cell growth in all three cell-lines on day 5 post-seeding after averaging the three trials. ErbB4 expression resulted in considerably stunted cell growth in the BT-20 and BT-549 cell lines, with no significant growth effect on the MDA-MB-468 tumor cells.

Migration assay

To assess whether ErbB4 will alter the motility of TNBC cells, a migration assay was performed evaluating BT-20, BT-549 and MDA-MB-468 tumor cell migration compared to the empty vector construct controls. Figure 4 depicts comparisons of cell migration across the three cell lines. According to our results, ErbB4 actually increased cell migration in the BT-20 cells and had no significant effect on motility in the other TNBC cell lines.

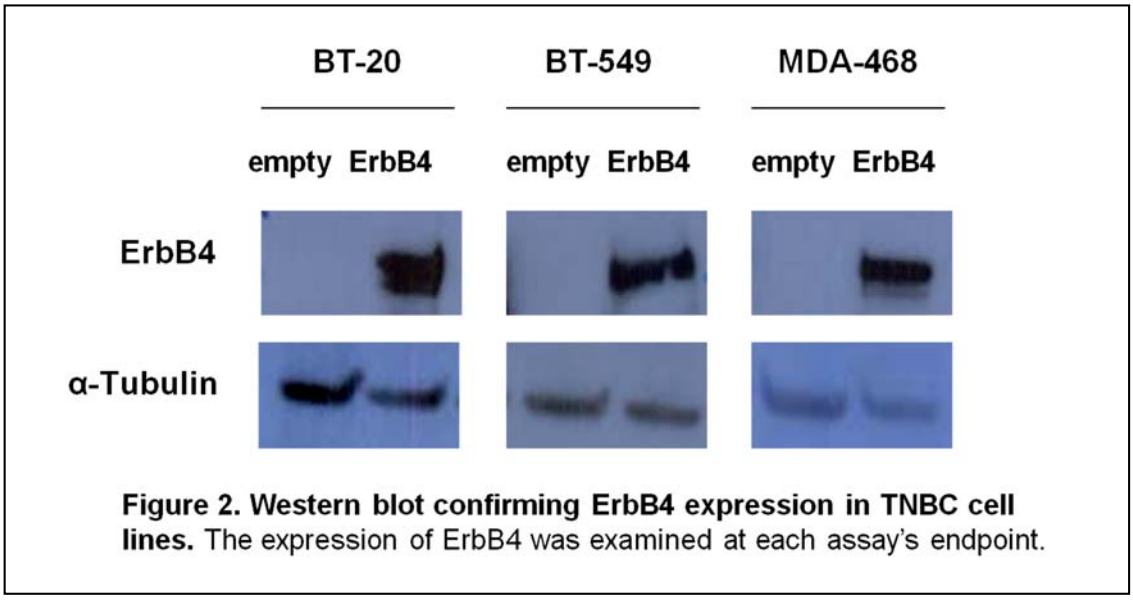

Figure 2. Western blot confirming ErbB4 expression in TNBC cell lines. The expression of ErbB4 was examined at each assay's endpoint.

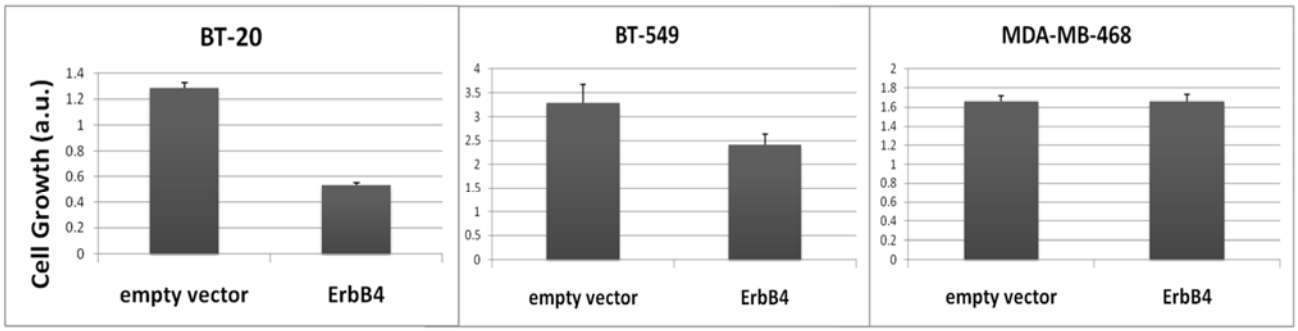

Figure 3. Graph of TNBC cell growth using SRB assay. These results depict normalized data for cell growth on day 5 post-seeding across the 3 TNBC cell lines tested. In the BT-20 and BT-549 cell lines, ErbB4 exhibited a growth inhibitory character, while in the MDA-MB-468 cell line, ErbB4 had no significant effect on cell growth.

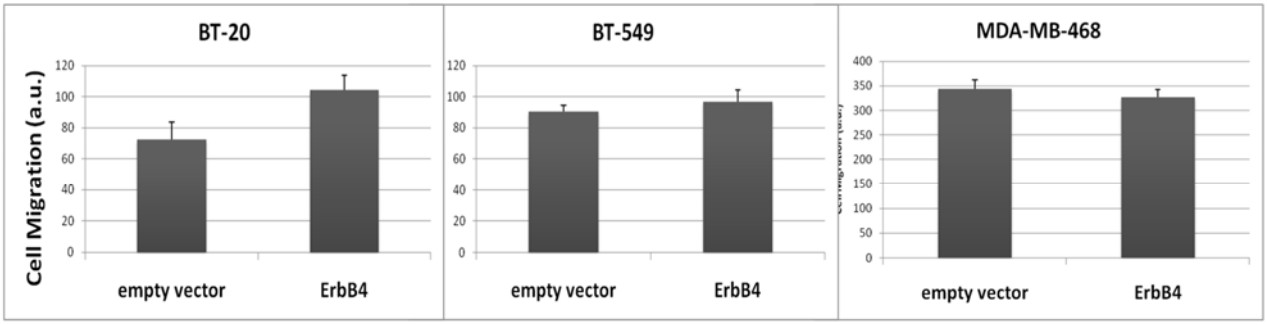

Figure 4. Graph of TNBC cell migration. These results depict cell migration approximately 24 h after seeding the cells in a transwell migration assay. ErbB4 enhanced migration in the BT-20 cell line, while resulting in no significant change in cell motility in the BT-549 and MDA-MB-468 cell lines.

Discussion

This study demonstrated that ErbB4 expression inhibited cell growth in two of three TNBC cell lines and, contrary to our hypothesis, enhanced cell migration in one subset of tumor cells with no significant change in the other cell lines. These findings corroborate previous studies describing the complexity and heterogeneity seen both in TNBC cells and within the intricate ErbB receptor tyrosine kinase signaling network. The observation that ErbB4 largely antagonized TNBC cell proliferation is also consistent with the sizable body of literature describing the protein's tumor-suppressor-like function. As one of few studies evaluating ErbB4 utilizing human TNBC parental cell lines, our results also reveal that the growth inhibiting effect of ErbB4 demonstrated in previous experiments performed in animal models are indeed transferrable to humans. It follows that the downregulation of this receptor may serve as a critical factor in lending TNBC its more aggressive features. Possible reasons for the differing responses to ErbB4 overexpression in the TNBC parental cell lines include the distinct receptor tyrosine kinase milieu each subset hosts. The relative quantities of the other ErbB receptors on the cell membrane as well as their respective agonists have repeatedly been shown to be critical in modulating the specificity of a signaling response. In fact, the sensitivity of the transduction cascade takes into account even spatial and temporal factors in receptor-receptor and receptor-ligand interactions which were beyond the scope of our study. In addition, differences in the assortment of downstream signaling molecules, e.g. WWOX, STAT5A, etc., between the cell lines may certainly contribute to the observed response variability. Another possibility is that ErbB4 may have different transcriptional coactivation potential in a nonparous versus a parous host due to global reorganization of chromatin structure caused by cycles of estrogen and progesterone[35]. Notably, the MDA-MB-468 parental cell line was obtained from a metastatic site demonstrating adenocarcinoma in a 51 year old Black woman whereas the BT-549 and BT-20 cell lines were derived from the primary breast tumors which in both cases were invasive ductal carcinomas in Caucasian women in their early 70s[36, 37]. An obstetrical history of these donors is not available but age, ethnicity, tumor subclassification and site of procurement serve as potential confounding variables. Additionally, a study by Xu et al. (2008) revealed considerable cytogenetic instability of the MDA-MB-468 cell line which may potentially

undermine use of these cells as a representative model. Agelopoulos et al. (2008) adds to this discovering multiple spontaneous fragile sites shown in FISH analysis which may influence cell heterogeneity.

Our study contains several important limitations. Firstly, tumor cell culture and growth was conducted on plastic surfaces. This charged, polarized surface may have the effect of independently promoting or retarding cell growth, thereby interfering with the integrity of the results. Furthermore, confirmation of ErbB4 expression in transfected cells was obtained without regard for subcellular localization. As the divergent effects on cellular responses varies depending on where the protein sequesters within the cell, results of this study may represent an oversimplification. Moreover, despite the clear utility of the SRB growth assay in determining cell density, the protocol lacks sensitivity in distinguishing between cell proliferation and cell death and is considerably influenced by plating efficiency. In addition, the relatively small sample size and number of trials limit the power of the study, thereby hindering the generalizability of our findings. It is also not certain whether ErbB4 levels gathered in our study reflects those found *in vivo*. Lastly, this study focused entirely on the ErbB4 receptor and did not investigate the quantity of the other ErbB proteins in the TNBC cells. This may result in overlooking potential modulators of outcome.

Future Directions

In the future, additional functional and mechanistic studies to further elucidate the role of ErbB4 in TNBC is warranted to pave the way to more clinically meaningful findings. These should include a more comprehensive analysis of all ErbB receptor family members and their ligands to clarify whether incorporating ErbB4 into clinical practice could provide additional information regarding the management of breast cancer. Moreover, a more complete understanding of the variables that give rise to the multiplicity of signal outcomes is in order. This would not only provide a means by which underexpressed or overactive ErbB receptors could be targeted, but could also be manipulated to achieve the desired effects of stimulation, such as ErbB induced apoptosis in receptor overexpressing cells.

Conclusions

Recent years has seen remarkable advances in our comprehension of TNBC, both clinically and molecularly. Given the relative aggressiveness of these tumors and the absence of targeted treatments, the identification of sites for therapeutic intervention is an urgency. The loss of ErbB4 is a frequent event in TNBC which occurs through various genomic mechanisms. In our study, the TNBC cell lines were transfected with either ERBB4 or empty vector expression constructs. GFP fluorescent microscopy as well as western blot confirmed the expression of ErbB4 in TNBC cell lines. Growth and migration assays were then conducted to compare ErbB4-transfected TNBC cells with cells expressing empty vector control construct. It was demonstrated that overexpression of ErbB4 enhanced migration of BT-20 cells, but yielded no significant difference in the migration of BT-549 or MDA-MB-468 cells compared to the empty vector control. Furthermore, ErbB4 over-expression inhibited growth of BT-20 and BT-549 cells, while causing no significant change in growth of MDA-MB-468 cells. Additional exploration of ErbB4 function in TNBC is required to investigate its potential role as a unique therapeutic target in TNBC.

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